

The University of Chicago

A METHOD FOR
THE STUDY OF THE HEMOGLOBIN-
OXYGEN EQUILIBRIUM

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
PHYSICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

1939

By
WILLIAM G. SMILEY

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INTRODUCTION

The equilibrium between hemoglobin and oxygen is of great interest both for physiology and for chemistry. The quantitative behavior of this reaction has been a subject of dispute for many years, and the question must still be considered open. The amount of oxygen absorbed by a hemoglobin solution at various pressures has been studied by numerous investigators,¹ but there has been a lack of agreement among their results. In part this is due to the nature of the problem--the instability of hemoglobin, the difficulty of preparing it in a state of definite purity, the uncertainty of its identity as a single chemical substance, and especially the great effect of impurities of many kinds on the equilibrium. In particular it has been found in this laboratory² that inorganic salts have a depressing effect on the oxygen absorption of hemoglobin which is a specific property of the particular salt, so that much of the work which has been done in buffered solutions is at best difficult to interpret. Also it appears³ that a further purification of hemoglobin results in a higher oxygen affinity than found by any previous investigators.

A part, however, of the difficulty in the study of this equilibrium arises from the lack of a satisfactory experimental method. Most workers have employed the method of passing a mixture of oxygen (at a known partial pressure) and some inert gas over the hemoglobin solution until equilibrium is reached and then analyzing the solution for oxyhemoglobin either spectroscopical-

¹J. Barcroft, "Respiratory Function of the Blood," Cambridge, 1925, Pt. 2, chaps. x-xii.

G. S. Adair, A. V. Bock, and N. Field, Jr., J. Biol. Chem., **63**, 529 (1925).

R. M. Ferry and A. A. Green, J. Biol. Chem., **81**, 175 (1929).

²A. E. Sidwell, Jr., R. H. Munch, E.S.G. Barron, and T. R. Hogness, J. Biol. Chem., **123**, 335 (1938).

³A. M. Altschul, A. E. Sidwell, Jr., and T. R. Hogness, J. Biol. Chem., **127**, 123 (1939).

ly¹ or by some chemical method such as that of Van Slyke.²

The former method has the disadvantage that the range is restricted, the results becoming very inaccurate for low values of oxygen absorption. The great disadvantage of the chemical method is that it destroys the hemoglobin, so that only one value can be obtained from a single sample. In view of the instability of hemoglobin and the effect of impurities, comparisons of different samples are bound to be inaccurate.

Another error of both these methods is due to the difficulty of estimating the partial pressure of oxygen accurately in a mixture in which it is present only in small concentration. In using a mixture of 0.1 mm. of oxygen and one atmosphere of nitrogen, for example, it is hardly possible to know the oxygen pressure with much precision. No one has even attempted to work below this pressure.

Unfortunately, these low pressures have become increasingly important since purification of hemoglobin increases the oxygen absorption at low pressures. Altschul, Sidwell, and Hogness,³ for instance, find that at pH 7.3 and 1 mm. oxygen pressure, the hemoglobin solution is 50 per cent saturated. Moreover, it is very desirable from the standpoint of theoretical interpretation to know the absorption-pressure curve at low pressures, since the limiting slope of this curve should be in agreement with equilibrium "constants" used at higher pressures.

It is surprising that so few workers have used the rather obvious method of determining the oxygen absorption directly by volume and pressure measurements alone. This method is capable of accuracy at low pressures and for small quantities; it is direct and unambiguous, and it does not destroy the hemoglobin, so that a series of measurements can be made on the same sample. Experiments of this sort were made by Bohr⁴ and more recently by

¹See for example Sidwell, Munch, Barron, and Hogness, op. cit.

²D. D. Van Slyke, J. Biol. Chem., 30, 347 (1917); Proc. Nat. Acad. Sci., 7, 229 (1921).

³Op. cit.

⁴C. Bohr, "Experimentale Untersuchungen über die Sauerstoffaufnahme des Blutfarbstoffes," Copenhagen, 1885. Cited by Ferry, J. Biol. Chem., 59, 297 (1924).

Ferry.¹

These workers did not attempt, however, to go to lower pressures than can be measured with an ordinary manometer. The lowest pressure reported by Ferry is 2.47 mm., which would correspond to not less than 50 per cent saturation in pure hemoglobin at neutral pH. His apparatus is, moreover, excessively complicated. It includes an equilibrator, a Töpler pump, a gas burette, a manometer, and a device for removing water vapor from the oxygen before it is measured. In the apparatus to be described in this paper the last-named device is dispensed with and the gas burette and Töpler pump are combined in one very simple device which also acts as a McLeod gauge.

In developing the method about to be described, the primary objective was to facilitate measurements at low pressures, but the method has been applied at pressures up to 35 mm. Although the writer was not familiar during this research with the work of Bohr and of Ferry mentioned above, the fundamental principle is the same. Pure oxygen is the only chemical reagent applied to the hemoglobin solution; all the measurements are carried out in a closed glass system; and the hemoglobin solution is not destroyed. It is believed that the method is capable of more accuracy than most of those used hitherto. The most striking difference from all previous work, however, is that measurements can be made at pressures as low as 0.01 mm. This will probably prove to be the most useful feature of the method.

EXPERIMENTAL

Theory of the method.--Essentially, the method consists in measuring the volume and pressure of pure oxygen in equilibrium with a hemoglobin solution, at various pressures and a constant temperature. The difference between the total amount of oxygen in the system and the amount present in the gas phase (given by the gas laws) is the amount in the solution. Measured quantities of oxygen may be introduced into or removed from the apparatus. Low pressures and small quantities of oxygen are measured by using the principle of the McLeod gauge.

In carrying out such measurements, the most serious dif-

¹R. M. Ferry, J. Biol. Chem., 59, 295 (1924).

difficulty is that the pressures it is desired to measure are often much smaller than the pressure of water vapor which is necessarily present above the solution. This difficulty is obviated by using a mercury manometer, both arms of which contain saturated water vapor at the same temperature. Since the pressure of the water vapor does not change on compression, the principle of the McLeod gauge may be used to measure pressures as low as 10^{-4} mm. of oxygen in the presence of 25 mm. of water. The presence of saturated water vapor in all parts of the apparatus also serves to minimize volume changes in the solution due to evaporation and condensation.

The apparatus.--Figure 1 is a schematic diagram of the apparatus. The entire system is made of Pyrex. From the bulb B mercury is drawn into the two manometer arms U and V. The height of the mercury columns is regulated in the usual way by changing the pressure in bulb B, air being admitted through a short piece of thermometer tubing and withdrawn with a water aspirator. The thermometer tubing reduces the flow sufficiently to facilitate accurate adjustment and to avoid any danger of forcing mercury into the stopcock S_2 .

The manometer arm U is closed during an experiment, the stopcock S_3 being opened only to equalize pressure when pumping out or admitting gas at atmospheric pressure. The cell W, attached to the arm U by a greased ground joint, contains water which supplies both sides of the manometer with saturated vapor. After water has been pumped out of arm V, the supply may be replenished by lowering the mercury below the junction of U and V. In order to keep the vapor pressure uniform, all that part of the apparatus shown enclosed in a dotted line is immersed in a water bath thermostatted to $\pm 0.001^\circ \text{C}$.--probably much better than is necessary.

The tube V acts not only as one arm of the manometer, but also as the closed arm of a McLeod gauge, as a gas burette for measuring quantities of gas added to or withdrawn from the solution, and as a Töpler pump for evacuating the whole apparatus. It is made of three sizes of tubing--40 mm., 8 mm., and 2 mm. in inside diameter, respectively. This gives a range of compression ratios which permits the measurement of pressures from 100 mm. to 0.01 mm. within ± 1 per cent (assuming heights of mercury columns are accurate to ± 0.1 mm.). The total volume of V is about 200 ml.

Through the stopcock S_2 the manometer can be connected

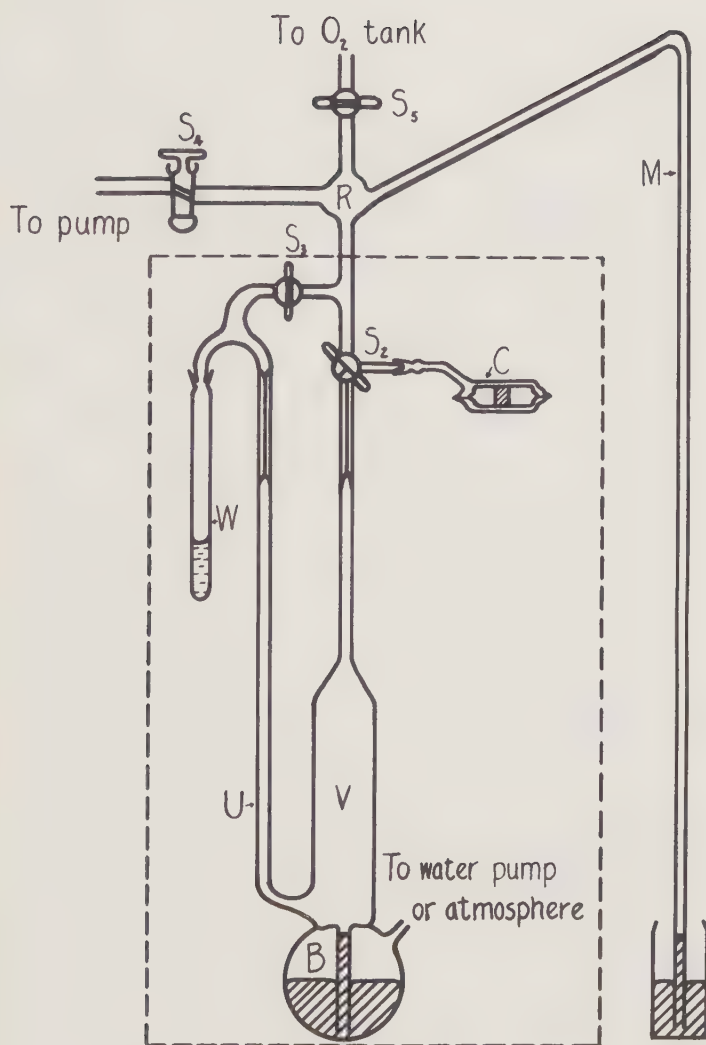


Figure 1

either to the reaction cell C or to the reservoir R, which in turn is connected either to the pumping system or to the oxygen supply. The manometer M is used to estimate the desired pressure of oxygen to be introduced into V, where it is measured exactly. The short arm of this manometer is a bottle of mercury, open to the air, which also acts as a safety valve through which oxygen escapes when the oxygen supply line is being swept out. The pumping system includes a mercury vapor pump, but in practice it was not used, a Cenco Hyvac pump being entirely adequate.

The cell C, attached to the apparatus by a greased ground joint, contains the hemoglobin solution. Its construction is shown in detail in Figure 2. The cell is so designed as to minimize the ratio of gas volume to solution volume while providing ample contact between solution and gas. The actual volume of the cell is about 11 ml., and it is convenient to use 4 ml. of solution. The solution is stirred by a rotary stirrer which is one of the most important features of the apparatus. The object is to bring the solution into equilibrium with the gas, prevent foaming, and avoid agitation which would destroy the hemoglobin. The stirrer is a glass cylinder, completely sealed, which fills most of the volume within the cell. It is mounted on two sharp tungsten points sealed into the ends of the cell and fitting into conical depressions ground in the ends of the cylinder. The cylinder is accurately balanced on these bearings and turns very easily.

The stirrer is turned by a rotating magnetic field, acting on a strip of iron fixed inside the cylinder, and generated by the circuit shown in Figure 3. A commutator, turned at 40 r.p.m. by a small motor with a worm gear drive, has two 67.5° sectors each connected to a slip ring. Direct current is fed to the sectors through the slip rings and by means of the four symmetrically placed brushes is converted into a (non-sinusoidal) two-phase alternating current. This is fed to the two windings of the electromagnet, which surrounds the cell C and is essentially a two-phase two-pole motor producing a field rotating (in 45° steps) at 40 r.p.m. If the resistances R are properly chosen, the stirrer turns steadily at the same rate. This stirring system proved very efficient in avoiding foaming while pumping out the solution and in reaching equilibrium without violent agitation.

The water cell W is also provided with an electromagnetic

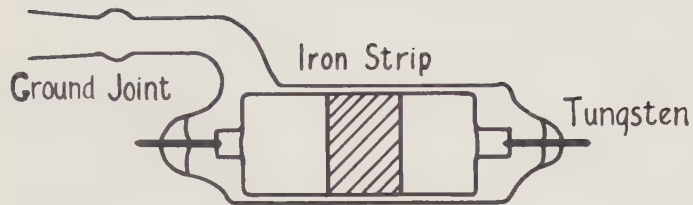


Figure 2

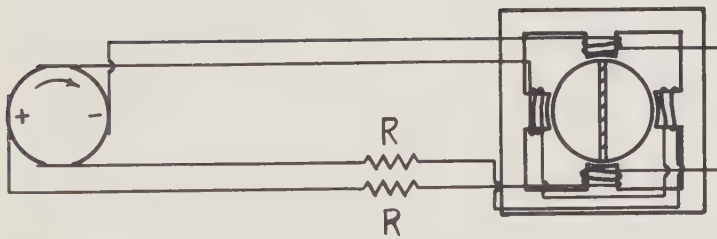


Figure 3

stirrer, an iron nail enclosed in a glass capsule and alternately lifted and dropped by a solenoid operated by a flasher button. This is used when pumping out the apparatus to hasten removal of air from the water.

Heights of the mercury columns are measured with a cathetometer. Readings can easily be checked within 0.1 mm. Before and after each experiment the cathetometer is set on a reference point on the apparatus (one of the glass hooks to which the cell C is attached by rubber bands) so that the height of the mercury columns is known relative to the apparatus itself and not merely to the cathetometer scale. Some difficulty was encountered in finding a lighting system which would make the mercury meniscus appear sharp and distinct at all levels. In the system finally adopted, a white screen (made of enameled brass) was placed behind the apparatus and illuminated by a 40-watt bulb at its base. The bulb is set in a mounting made waterproof by paraffin and equipped with a reflector which allows the whole screen to be illuminated but prevents direct light from reaching the observer. Against this white background, with all light coming from below and behind, the mercury meniscus is silhouetted quite clearly, and readings can easily be reproduced within 0.1 mm. even in the 40 mm. tube.

Calibration.--In order to measure the quantity of gas present in the tube V, and also to use it as a McLeod gauge, it is necessary to know the volume of the space above the mercury in V as a function of the height of the mercury. This function was determined by the application of Boyle's law. In each of the three sections of the tube V there is a linear relationship

$$V = a(b-h) \quad (1)$$

connecting the volume V with the height h of the mercury. The constants a and b are different in the three sections. For a given quantity of gas in V we may apply Boyle's law and write

$$p(b-h) = \text{const.} \quad (2)$$

where the constant depends on the temperature, the quantity of gas, and the value of a. The manometer arm U was evacuated as described later, a small quantity of air was introduced into V, and a series of values of p and h were measured, the amount of gas being such that p could be measured directly as the difference in level between U and V. (In the lowest section of V, it was necessary to make a correction of 0.6 mm. for capillary depression; in the upper two sections the corresponding part of U is made of

the same size of tubing, making a correction unnecessary.) By simultaneous solution of the resulting equations (2), b was determined in each section of the tube. The relative values of the constants a were determined by using a quantity of air such that p and h could be measured in two different sections, so that

$$pa(b-h) = p'a'(b'-h') \quad (3)$$

The volume is thus determined as a function of height except for an unknown constant factor. The volume c of the space above the solution in cell C (including the stopcock S_2) was determined in terms of this same constant by filling it with gas at a measured pressure, evacuating V, connecting V and C, and measuring several values of v and h . During this determination the cell C contained 4 ml. of water, the same volume as the solution used during an experiment. The volume of the stopcock alone can also be calculated from this experiment, but in practice it is seldom necessary to use it.

Thus the relative volume of the essential parts of the apparatus was determined with an accuracy of ± 1 per cent. The absolute volume could then be determined by filling cell C up to the ground glass with water from a burette, estimating the volume of the connecting tubing, and subtracting 4 ml. to obtain c . This determination is less accurate than the others, but it affects only the measurement of absolute quantities of gas; it has no effect on the ratio of gas absorbed at a given pressure to maximum absorption, which is the quantity of greatest theoretical interest.

The three equations (1) were determined with dry air, the cell W being empty, and in the presence of water vapor, i.e., under the conditions of an actual experiment. The results of the two calibrations checked within the experimental error of 1 per cent, except in the case of the 2 mm. capillary, for which the "wet" calibration gave a value of the cross-section corresponding to a film of water 0.1 mm. thick on the walls of the capillary. The thickness of this film, which could be plainly seen in the cathetometer telescope, is no doubt due to grease from the stopcock S_2 . The same cause is no doubt responsible for irregularities in pressure measurements in the capillary, which were observed in the wet but not in the dry calibration. Also the volume of the capillary is uncertain because of the water condensed on the mercury. As the mercury column is raised, the water vapor in V condenses; most of it remains trapped between the mercury and

the glass, but at times enough condenses in the capillary to form a layer 1 mm. or more in height. After several attempts to correct for the volume of this water, it was found that as consistent results as any were obtained by ignoring it and finding the empirical equation for the apparent volume on the basis of the mercury meniscus alone. It turns out that the errors due to this irregular behavior are insignificant compared with other factors, at the low pressures at which the capillary is useful.

Measurements on hemoglobin.--In making an experiment, the following procedure was followed: 4 ml. of hemoglobin solution, taken from a cold room at $0 - 3^{\circ}$ C., was pipetted into the cell C and the latter attached to the apparatus. The thermostat was refilled with water (it is necessary to drain it to remove the cell) and heated up to 25° C. with an auxiliary 1,000-watt heater. While the thermostat was warming up, the manometer was evacuated as follows: with stopcocks S_2 and S_3 open, the bulk of the air was rapidly pumped out; the stopcocks were closed and the mercury lowered below the junction of U and V; the mercury was raised as far as practicable, stopcock S_2 opened and the air pumped out of V. During the pumping the mercury was raised into the capillary, so that practically all the air was removed; then stopcock S_2 was closed and the mercury lowered below the junction. By repeating this process, the air was removed with a minimum loss of water vapor. Since the mercury column acts as in a Töpler pump, compressing the air and forcing it out of the apparatus, it was easily possible to obtain a "vacuum" of 10^{-4} mm. (exclusive of water vapor) with the Hyvac pump. While the air was being pumped out, the water in W was stirred, in order to remove as much air as possible; afterwards the stirring was stopped, so that any remaining air would escape slowly. In this way it was easy to keep the pressure in U well below 0.1 mm., which is all that is necessary.

When the manometer was evacuated and the thermostat operating, the air was removed from cell C by a similar process. With V saturated with water vapor the mercury was raised almost to the 8 mm. tube and V and C were connected. The mercury was slowly lowered, with constant stirring of the hemoglobin solution to prevent foaming. After expansion to the maximum volume of V, S_2 was closed, the mercury raised and the air pumped out as before, S_2 was then closed and the mercury was lowered until the water trapped between the mercury and the glass evaporated and again

saturated the volume with vapor. V was then again connected with C and the cycle repeated. The process was continued until it was found (by compressing the gas removed from C into the capillary) that the pressure in C had been lowered to well below 0.01 mm. and until no rise in pressure occurred on stirring the solution for 8 - 10 minutes. (As appears from the results, the amount of gas remaining in the solution at this pressure is negligible compared to the amount to be added.) Since V was always saturated with water vapor when connected to C, the tendency to pump water out of the cell was minimized.

The stopcock to the pump (S_4 , Fig. 1) was now closed and the stopcock to the oxygen line opened. Ordinary tank oxygen (99.5 per cent) was used. The tank was connected to the apparatus by rubber tubing, but a large glass tube was included in the system to increase the volume so that the pressure would not fall below atmospheric on connecting to the reservoir R. The system was swept out by passing oxygen into R and out through the manometer M, the pressure in the rubber tubing being always above atmospheric. Stopcock S_2 was then closed, R was evacuated through stopcock S_4 , and oxygen admitted to a pressure of about 5 cm. With the mercury in V sufficiently low that the volume of the stopcock could be neglected, R was connected to V and the stopcock S_2 immediately closed again. The mercury was lowered (but not below the junction) to replace any water lost in this process, raised into the 8 mm. section, and the volume and pressure (about 100 mm.) were measured. C and V were connected and the solution stirred until the pressure became constant at about 35 - 40 mm., which took about 15 - 20 minutes. The pressure and volume were then measured again. The quantity of oxygen in the gas phase being known before and after the opening of the stopcock, the difference is the amount of oxygen absorbed by the solution. The amount absorbed at 35 - 40 mm. was taken as the maximum. This quantity was also determined independently by another method as described later.

With V and C still connected, the mercury was lowered, the solution and gas brought into equilibrium by stirring, the mercury level was read to determine the volume, and after closing S_2 the mercury was raised until the pressure and volume could be read accurately. Thus the pressure at equilibrium and the amount of oxygen in the gas phase were known and the amount absorbed

could be determined as before. When a measurement had been made at the lowest pressure available by this method, V was pumped out, the water replaced as usual, and the measurements continued at still lower pressures. Since the amount of oxygen removed had been measured, the total amount present was still known. This process was continued until pressures below 0.01 mm. were reached. In general, the gas and solution were equilibrated for at least 15 minutes. (Since the bulb B is thermostatted, the mercury level could be kept constant during an equilibration.) At the lower pressures, since the pressure could not be read continuously, it was difficult to know when equilibrium had been reached. Several measurements were therefore checked by returning as nearly as possible to the previous level, stirring for 8 - 10 minutes, and re-reading the pressure. The details of calculations and the results of these measurements will be discussed later.

Finally the total absorption at 35 - 40 mm. was measured again by the same procedure as above.

Hemoglobin solution.--The hemoglobin solution used in these measurements was prepared approximately according to the method of Altschul, Sidwell, and Hogness.¹ The erythrocytes of horse blood were concentrated by decantation, washed four times with 1 per cent sodium chloride solution, and treated with water and toluene. The blood was treated with alumina cream, dialyzed for 48 hours, again treated with alumina cream and filtered. Just before use the solution was centrifuged at high speed and a clear solution was obtained. The pH of the solution (measured with a glass electrode after completing a run) was 7.16.

DISCUSSION

Calculations and results.--The results obtained on a hemoglobin solution with the apparatus described above are presented in Table 1 and Figure 4. The figures in the second column are in units of pressure (in millimeters) times volume (in milliliters). At the experimental temperature, 25° C., one such unit is 5.38×10^{-8} mols.

In the first line of the table is the maximum absorption, determined at the start of the experiment as indicated above. It

¹Op. cit.

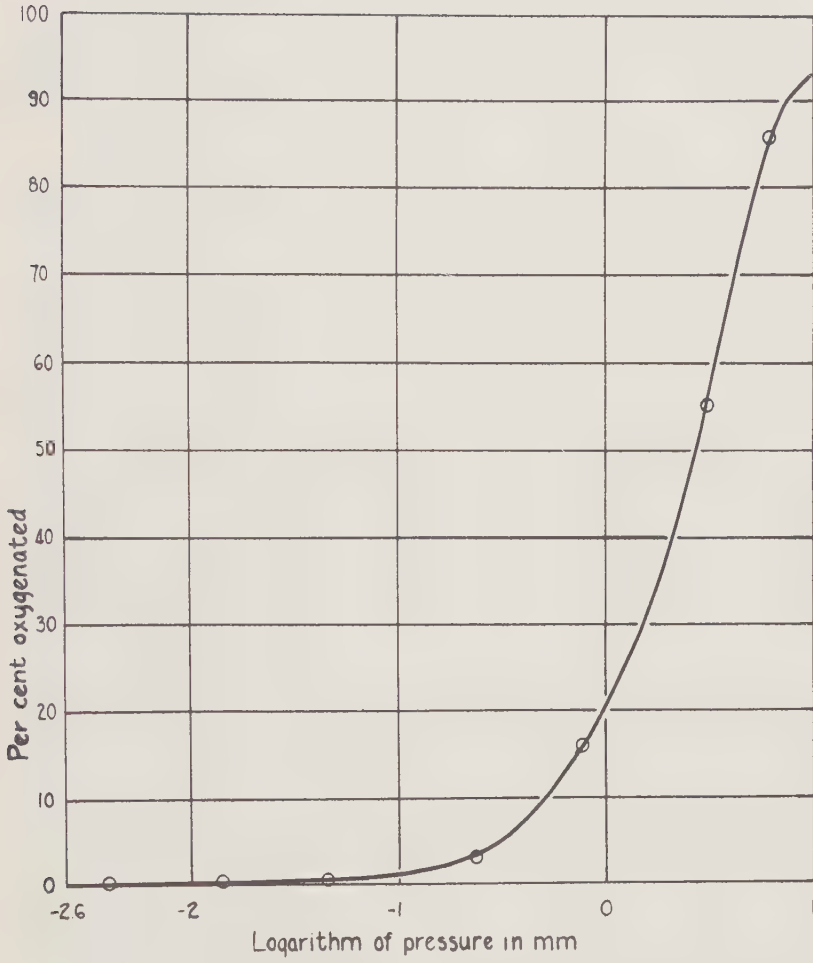


Figure 4

TABLE 1
OXYGEN ABSORPTION OF DIALYZED HEMOGLOBIN AT 25° C.

Pressure in mm. Hg	Amount Absorbed by 4 ml. Solution	Per cent of Maximum
35.0	303	(101)
0.004	0.3	0.10
0.014	0.8	0.27
0.043	3.0	1.00
0.235	8.9	2.97
0.773	47	15.7
3.14	164	54.7
6.26	256	85.3
35.0	300	100.0
32.4	311	(104)

is possible to calculate all the other values in the same way, as the difference between the amount added and the amount removed. However, this method introduces into all the values of the absorption an uncertainty equal to the error (at least one unit) in the determination of the total amount added. The measurements at lower pressures involve a much lower error and therefore it appeared desirable to calculate the absorption at these pressures in a manner which would be independent of values at higher pressures. This was done by calculating the absorption at a given pressure as the total amount of oxygen removed from the solution below this pressure, plus the amount remaining in solution at the lowest pressure measured (0.004 mm.). This last quantity was, of course, not measured and must be estimated, which was done as follows: The absorptions at the lowest three pressures were calculated on the assumption that the lowest of them was zero, and the resulting values were plotted against the pressure. As would be predicted at low pressures by any of the usual theories, they fell on a straight line. This line was extrapolated to the axis of zero pressure and the resulting point taken as the true zero of oxygen absorption. Thus the value of 0.3 at 0.004 mm. was obtained. In view of the uncertainty of the extrapolation, it was not thought justified to express this value (and therefore all

the others, which depend on it) to more than one decimal place.

All the values in the second column of Table 1, except the first and the last, were calculated in this way. The third column gives the oxygen absorption as a percentage of the maximum 300. Since this maximum was determined as the total quantity of oxygen removed from the solution, it is independent of the value measured directly (303); the agreement between the two is an indication of the accuracy of the method.

The maximum absorption was also measured at the end of the run. The value so obtained, given in the last line of Table 1, is higher than can be explained by the error of measurement. It can, however, be plausibly accounted for by supposing that, in spite of the precautions taken, the solution lost some water during the course of the run. It had in fact been observed that over a long period of time water would distil from one part of the apparatus to another, in spite of the uniform temperature. A decrease in volume of 0.3 ml. (causing a corresponding increase in the volume *c*) would be sufficient to explain the discrepancy, yet would have no effect on the measurements at lower pressures. This is true because the amount of oxygen in the gas phase in cell C, at all pressures except the highest used, is small compared to the amount of the solution.

The values in Table 1 have been corrected for the solubility of oxygen in water. The correction is appreciable only at the two highest pressures.

Possible error.--In all probability the largest error in these results is due to failure to reach equilibrium. It should be pointed out that this would affect only the interpretation of the particular value in question, and would not interfere with the "bookkeeping" by which the absorption is determined at other pressures. As mentioned above, checks were made on some of the measurements by allowing further time for equilibration and remeasuring. The changes thus found were of the order of 5 per cent, indicating that equilibrium had not quite been reached, but also that the final values were very nearly correct. The results at 0.043, 0.235, and 6.26 mm. were checked in this way. It is probable that the others may be about 5 per cent high from this cause. At 0.01 mm., however, previous experiments showed that 15 minutes is ample to reach equilibrium. At low pressures in general this error is less than that due to the uncertainty in estimating the residual

oxygen in solution. This error may perhaps be reduced by pushing the measurements to still lower pressures, but even as it stands the method is good to $\pm 5 \times 10^{-9}$ mols. of oxygen and perhaps $\pm 2 \times 10^{-4}$ mm. pressure. In the solution studied this is ± 0.03 per cent of the total, so that figures below 1 per cent are accurate enough to have a definite meaning.

The problem of reaching equilibrium needs further study. It seems very probable that an equilibration period of 30 minutes would be ample, and if this were found to be so, the time-consuming checks could be eliminated.

Stability of hemoglobin.--In this connection it may be said that the need for haste in dealing with hemoglobin solutions may not be as great as has been supposed. By the procedure outlined above, the hemoglobin is largely in the reduced state during most of the run, and under these conditions there is no evidence of great instability. The high value for the absorption found at the end of the run, while it could be explained by a loss of volume, could not possibly be accounted for on the assumption that a considerable fraction of the hemoglobin became inactive during the run. The check between the amount of oxygen added and the amount removed is a further indication of stability. Ferry,¹ using a similar method, found that when he returned to high pressures after working at low pressures, the activity had decreased considerably. But this phenomenon could be explained by a failure to reach equilibrium as well as by the formation of methemoglobin.

Another indication of the stability of reduced hemoglobin, and also of the instability of the oxygenated form, is given by the following facts: The hemoglobin solution was kept in the oxygenated form in a cold room at $0 - 3^{\circ}$ C. A sample was removed, a run made on it, and after 24 hours at room temperature, most of that time in the reduced form, its oxygen uptake was found to be 385. After another 24 hours another sample was removed from the cold room; after about 6 hours its oxygen uptake was 303, and 5 hours later it was, if anything, higher. That is, the hemoglobin apparently lost more activity in the cold room, in the oxygenated form, in 48 hours, than at room temperature, in the reduced form, in 18 hours. Either the effect of temperature on the stability of

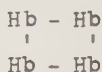
¹Op. cit.

hemoglobin is much less than is supposed, or the reduced form is much more stable than the oxygenated form. These results would indicate that it is desirable to keep hemoglobin in the reduced form.

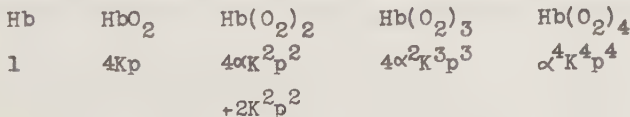
Advantages of the method.--The stability of reduced hemoglobin is important because it makes possible one of the most valuable features of the method; the determination of an entire absorption curve on a single 4-ml. sample. This not only means a great saving in time and materials, but also insures consistent results. The purely physical method, and the avoidance of all foreign substances, have obvious advantages in simplifying the situation.

The greatest value of the method, however, will probably be in the low pressure range to which other methods are not applicable. The possible uses of data at low pressures will be suggested in the next section. It has already been pointed out that a method valid below 1 mm. is a necessity in dealing with pure hemoglobin solutions.

Comparison with theory.--The measurements described here were of a preliminary nature and intended primarily to test the feasibility of the method. Too much stress should not be placed on the results, but it is interesting to compare them with a theoretical equation--that derived by Pauling.¹ This equation is developed on the assumption that each heme group of the hemoglobin molecule interacts with two others thus:



in such a way that the free energy decrease of adding oxygen to two interacting groups is greater than that of adding oxygen to two non-interacting groups by an amount $RT \ln \alpha$. If $RT \ln K$ is the free energy change of adding one oxygen, the relative amount of each molecular species present may be calculated by the mass law:

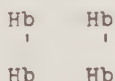


¹L. Pauling, Proc. Nat. Acad. Sci., 21, 186 (1935).

The factors of 4 and 2 are statistical weights resulting from the different ways each molecular species may be formed. The maximum amount of oxygen the solution can absorb is proportional to four times the sum of all these quantities, while the amount actually absorbed is proportional to the amount of HbO_2 plus twice the amount of $\text{Hb}(\text{O}_2)_2$, etc. Thus the fraction oxygenated is:

$$y = \frac{Kp + (2\alpha + 1) K^2 p^2 + 3\alpha^2 K^3 p^3 + \alpha^4 K^4 p^4}{1 + 4Kp + (4\alpha + 2) K^2 p^2 + 4\alpha^2 K^3 p^3 + \alpha^4 K^4 p^4} \quad (4)$$

If one assumes that each heme interacts with only one other:



one obtains the simpler equation:

$$y = \frac{Kp + \alpha K^2 p^2}{1 - 2Kp + \alpha K^2 p^2} \quad (5)$$

It has been found recently in this laboratory that equation (4) can be fitted to most of the data in the literature, and the results seem to indicate that α is lower, i.e., there is less interaction, the more free the solution is from salts, so that it seems likely that pure hemoglobin would show no interaction and would obey the simple mass law:

$$y = \frac{Kp}{1 + Kp} \quad (6)$$

When equation (4) is applied to the present data at higher pressures, $\alpha = 3$ seems to give the most constant K , as may be seen from the first row of Table 2. However, equation (5) fits the data equally well (second row of Table 2) with $\alpha = 10$.

TABLE 2

y	0.15	0.50	0.80
K(eqn. (4), $\alpha = 3$)	0.14	0.13	0.16
K(eqn. (5), $\alpha = 10$)	0.13	0.12	0.15

As must necessarily happen if the two equations fit equally well, both give approximately the same value of K, say 0.14.

In order to test the equations at lower pressures, one observes that both equations (4) and (5) give a limiting slope equal to K for y plotted against p. This limiting slope can be calculated from our low-pressure data as shown in Table 3.

TABLE 3

P	y/p
0.004	0.25
0.014	0.19
0.043	0.23
0.235	0.13
0.773	0.20

The point at 0.235 mm. deviates from the average much more than a reasonable estimate of the experimental error. Further work will be needed to decide whether the curve really has an inflection in this neighborhood or not. In any case, however, it is evident that the low pressure data do not fit the theoretical equations with the same constants which fit at higher pressures. It would be premature to draw definite conclusions from this preliminary work, but it illustrates the value of low-pressure data such as the present method provides.

SUMMARY

A method has been developed for studying the absorption of oxygen by hemoglobin solutions, as a function of pressure, at pressures down to 0.01 mm.

It is believed that this method is capable of greater accuracy than those commonly used.

The method permits an entire absorption curve to be determined from a single 4-ml. sample.

Some preliminary results are given.

